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ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -  
Kibbutz Ein Hamifratz, Mobile Post 25210,  
ISRAEL

Phone: + 972 52 3965809

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## Reproductive Characteristics and Egg Development in Flounder (*Pleuronectes flesus luscus*) in the Southern Black Sea

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(Received 13.8.07, Accepted 9.9.07)

Key words: flounder, *Pleuronectes flesus luscus*, fertilization, fecundity, egg diameter, egg development

### Abstract

Spawning time, total fecundity, egg size, fertilization and hatching rates, and egg development of the flounder, *Pleuronectes flesus luscus*, were investigated in six wild female broodstock (mean wt 394.4±226.8 g). Spawning lasted 33 days from December 29 to January 30. Mean total fecundity was 171.4±109 x 10<sup>3</sup> eggs per female. Newly ovulated eggs were spherical and buoyant, with a diameter of 1.075-1.213 mm (avg 1.156±0.025 mm), a colorless transparent chorion, a slightly yellowish unsegmented yolk, and a narrow perivitelline space, without an oil globule. Fertilization and hatching rates were 17.2±15.7% and 51.5±27.6%, respectively. Hatching occurred after 117 h of incubation at 9.8-11°C. There were variations in egg size between batches, with the size tending to decrease during the spawning season ( $p<0.05$ ).

### Introduction

The flounder, *Pleuronectes flesus luscus*, is a Pleuronectidae teleost that inhabits coastal and brackish waters in western Europe and from the White Sea to the Mediterranean and the Black Seas (Nielsen, 1986). Many flatfish species are commercially exploited by fisheries along the southern coast of the Black Sea but are not yet produced in aquaculture. In Turkey, turbot (*Psetta maxima*) and flounder

(*P. flesus luscus*) are important commercial flatfish species that are being considered for aquaculture. Although seed production techniques for *P. maxima* have been established (Ciftci et al., 2002), research on aquaculture of *P. flesus luscus* is limited to larvae rearing (Sahin, 2000) and adaptation and feeding of wild-caught flounder juveniles in aquaculture conditions (Ergun and Yalcin, 2006).

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For proper broodstock management of *P. flesus luscus*, information on its reproductive biology is required. Therefore, in the present study, *P. flesus luscus* broodstock, captured by trawl nets and adapted to culture conditions, were studied and its reproductive characteristics and egg development are herein reported.

#### Materials and Methods

Six females (25.2-42.0 cm in total length; mean wt 394.4±226.8 g) and seven males (18.4-24.0 cm TL) were collected with a trawl net by the research vessel of the Central Fisheries Research Institute (CFRI) during a survey conducted once a week at a depth of 5-70 m off Trabzon, Turkey, during December 2006. Upon arrival at the CFRI, fish were examined and put into a quarantine tank for observation and disease treatment. Fish were bathed in formaldehyde (100 ppm, 1 h) and furazolidone (20 ppm, 1 h) to prevent bacterial disease.

After two weeks of acclimation, fish were individually tagged and stocked at 3-4 fish per m<sup>2</sup> in a maturation tank (1 x 2 x 0.5 m). Light intensity was kept around 100 lux using fluorescent bulbs at night and ambient conditions during daytime. The daily water exchange averaged 900% a day. Aeration was provided by two air stones with a total output of 4 l/min. During spawning, the water temperature was maintained at 9.8-11.0°C (avg 10.1°C). The broodstock were fed whiting cut into 2-3 pieces. Feed was provided gradually until satiation.

Gonad maturation in males was examined microscopically. One drop of seawater was placed on a glass slide to which a small amount of milt was added. Sperm activity was examined under a microscope at 100 x magnification. Viable sperm were characterized by whirl-like movement after seawater and milt were stirred. Gonad maturation in females was evaluated by sampling a small amount of eggs with a canula. These were transferred to a glass slide and measured under 40 x magnification. Females with at least 100 oocytes larger than 0.4 mm in mean diameter were considered ready to spawn (Ciftci et al., 2002).

Males with viable milt and females ready for spawning received hormonal injections. Males were injected with a mixture of human chorionic gonadotropin (HCG) and white salmon pituitary gland (WSPG; 100 IU/kg fish). Females were injected with a pelletized luteinizing hormone-releasing analogue (LHRH-a; 100 µg/kg fish; Berlinsky et al., 1996).

Semen was routinely collected from males with a syringe with a silicone tube (1.5 mm, inner diameter) and stored in an icebox until insemination (within 1 h). The dry method was used for fertilization. Eggs from a single female were stripped into a dry plastic container (0.6 l), semen from two males was added (Berlinsky et al., 1996), and the two were mixed with a feather. Afterwards, small quantities of seawater were gradually added. Eggs were kept about 10 min in the mixing container to ensure fertilization. Eggs were incubated as described by Sahin (2000) except that the incubating temperature ranged 9.8-11.0°C.

Data were recorded for weight and length of spawners, spawning time, total fecundity (number of eggs/female), relative fecundity (eggs/kg female), egg diameter, fertilization rate (at the 4-cell stage, 4 h after fertilization), hatching rate (ratio of hatched larvae to total number of eggs), and development of egg stages. Data were analyzed using Minitab statistical software, and means and differences at the 5% level were considered significant.

#### Results

The spawning season commenced on December 29 and continued to January 30 (33 days). During 65 hand-stripping trials, a total of 5.11 ml milt from seven males and 1023.4 g eggs from six females were obtained. There were approximately 829-1250 eggs (avg 1005±122) in one gram. The hatching time at 10.1°C was 117 h, but at higher temperatures the hatching time dropped to 103 h at 11.3°C, 98 h at 12.4°C, and 77.5 h at 13.5°C. The relationship between hatching time (D) and temperature (T) was determined according to the formula  $D = 411.9 T^{-127.02}$  ( $r = 0.967$ ; Hamel et al.,

1997). Reproductive parameters are summarized in Table 1.

Fig. 1 shows the weight of eggs in each 24-h batch from each female. Female E, which was stripped 21 times, had the highest fecundity while female F had the lowest (Fig 2). Both total fecundity (TF) and relative fecundity (RF) were linearly related to body weight (BW), as determined by the formulae:  $TF = 243.56 BW + 75368$  ( $r = 0.5044$ ) and  $RF = 90322 BW + 55856$  ( $r = 0.494$ ).

Egg fertilization rates varied 0-59.2% (mean  $17.2 \pm 15.7\%$ ) and the total weight of the fertilized eggs was 175.2 g. The hatching rate ranged 0-93.2% (mean  $51.5 \pm 27.6\%$ ).

Newly ovulated eggs were buoyant and spherical, measured 1.075-1.213 mm (avg  $1.156 \pm 0.025$  mm) in diameter, had a colorless transparent chorion, slightly yellowish and unsegmented yolk, and no oil globule. The perivitelline space was narrow. There were black pigments on the embryo. Embryonic development is shown in Fig. 3.

### Discussion

Fecundity in *P. flesus luscus* ( $171.4 \pm 109 \times 10^3$  total fecundity;  $486.6 \pm 383 \times 10^3$  relative fecundity) appears to be lower than in other flounders. The mean fecundity of sand flounder (*Rhombosolea plebeia*) ranged 100-500  $\times 10^3$  eggs for fish of 18-30 cm while that of yellow-belly flounder (*R. leporina*) varied 250-

$1,250 \times 10^3$  eggs for fish of 30-45 cm (Colman, 1973). Fecundity in *Paralichthys orbignyanus* was 240-280  $\times 10^3$  eggs/kg for fish weighing 1.78-2.86 kg as reported by Cerqueira et al. (1997) and 185-399  $\times 10^3$  eggs/kg as reported by Bambill et al. (2006). Mean fecundity in southern flounder (*P. lethostigma*) was 735  $\times 10^3$  eggs/female for females weighing 1.12 kg (Watanabe et al., 2000) and 230-1,000  $\times 10^3$  eggs/female for females weighing 1.2-2.9 kg (Smith and Denson, 2000). These differences are not contradictory considering that broodstock size, age, and genotype, as well as daily and seasonal feeding rates, can influence the number of eggs produced (Bromage, 1996).

Fecundity in *P. flesus luscus* greatly varied with size but the average total and relative fecundity in this study had a linear relationship with weight. There is a general positive exponential relationship between fish size and number of eggs (Jenning et al., 2001). In most flatfish species, fecundity is positively related to age (Bagenal, 1966).

Flounder is a batch-spawning flatfish. Studies of the ovulatory periodicity of the flounder indicate that a one-day interval may characterize a regular ovulation pattern. According to our findings, females produced 6-21 batches for about a month and batch fecundity was usually within a range of 2-44  $\times 10^3$  eggs.

Table 1. Reproductive variables of flounder (*Pleuronectes flesus luscus*) broodstock.

| Variable                      | n   | Minimum | Maximum | Mean $\pm$ SD     |
|-------------------------------|-----|---------|---------|-------------------|
| Total length (cm)             | 6   | 25.2    | 42.0    | 30.6 $\pm$ 6.04   |
| Standard length (cm)          | 6   | 20.8    | 34.5    | 25.0 $\pm$ 4.97   |
| Body weight (g)               | 6   | 218.4   | 840.6   | 394.4 $\pm$ 226.8 |
| Total fecundity ( $10^3$ )    | 6   | 79.1    | 318.1   | 171.4 $\pm$ 109   |
| Relative fecundity ( $10^3$ ) | 6   | 246.1   | 1262.7  | 486.6 $\pm$ 383   |
| Egg diameter (mm)             | 203 | 1.075   | 1.213   | 1.156 $\pm$ 0.025 |
| Fertilization rate (%)        | 38  | 0       | 59.2    | 17.2 $\pm$ 15.7   |
| Hatching rate (%)             | 28  | 0       | 93.2    | 51.5 $\pm$ 27.6   |

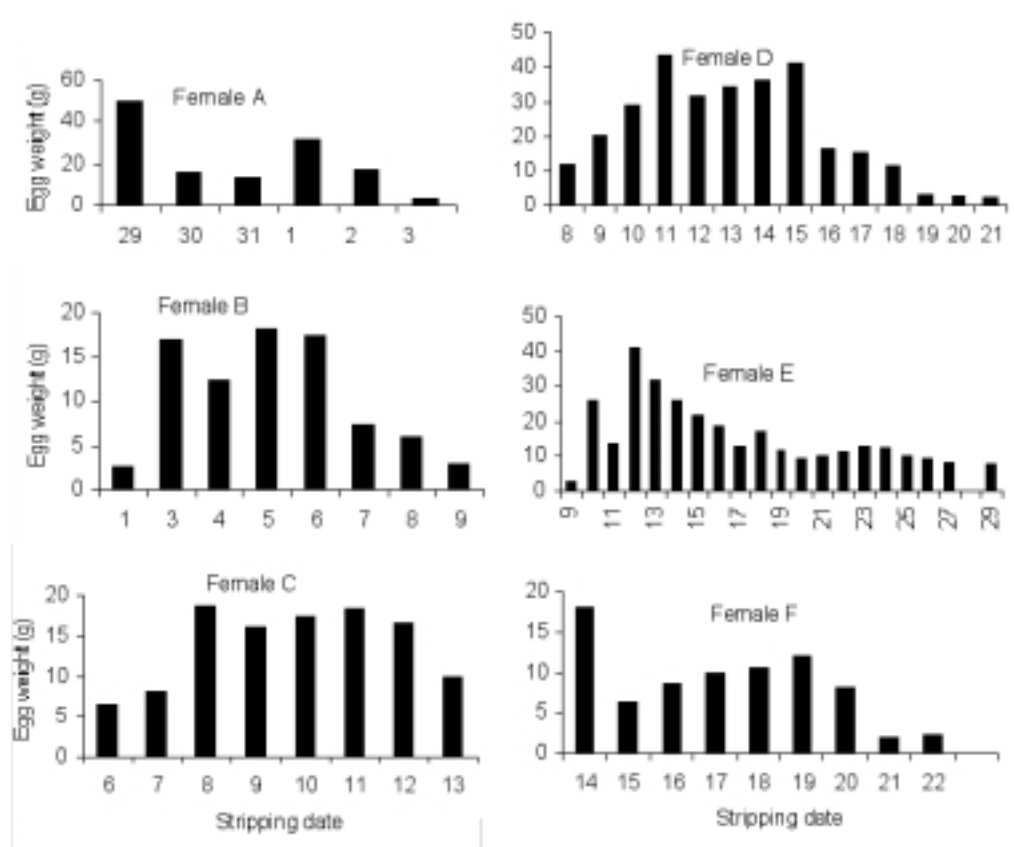


Fig. 1. Weight of eggs in 24-h batches from six female flounder (*Pleuronectes flesus luscus*) from December 29 to January 29.

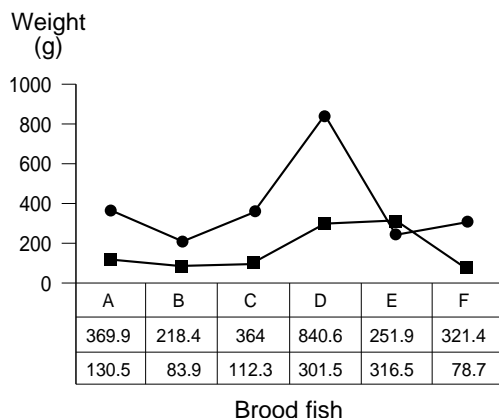


Fig. 2. Egg weight (■) and body weight (●) of six female flounders (*Pleuronectes flesus luscus*).

The variability in fertilization success in our study may be related to egg quality. Over-ripening is a significant determinant of egg quality in many fish species (Bromage, et al. 1994). The fertilization rate depends primarily on the time after ovulation (Koya et al., 1994). Fertilization rates of Black Sea turbot eggs decreased from 90-95% to 0% following a 24-h delay between ovulation and stripping (Maslova, 2002). In our study, the minimum time between two strippings was 24 h. Therefore, over-ripening and a low fertilization rate would be expected.

Incubation temperature and its variability from fertilization to hatching are probably the main factors controlling the duration of early development stages of fish embryos. In the

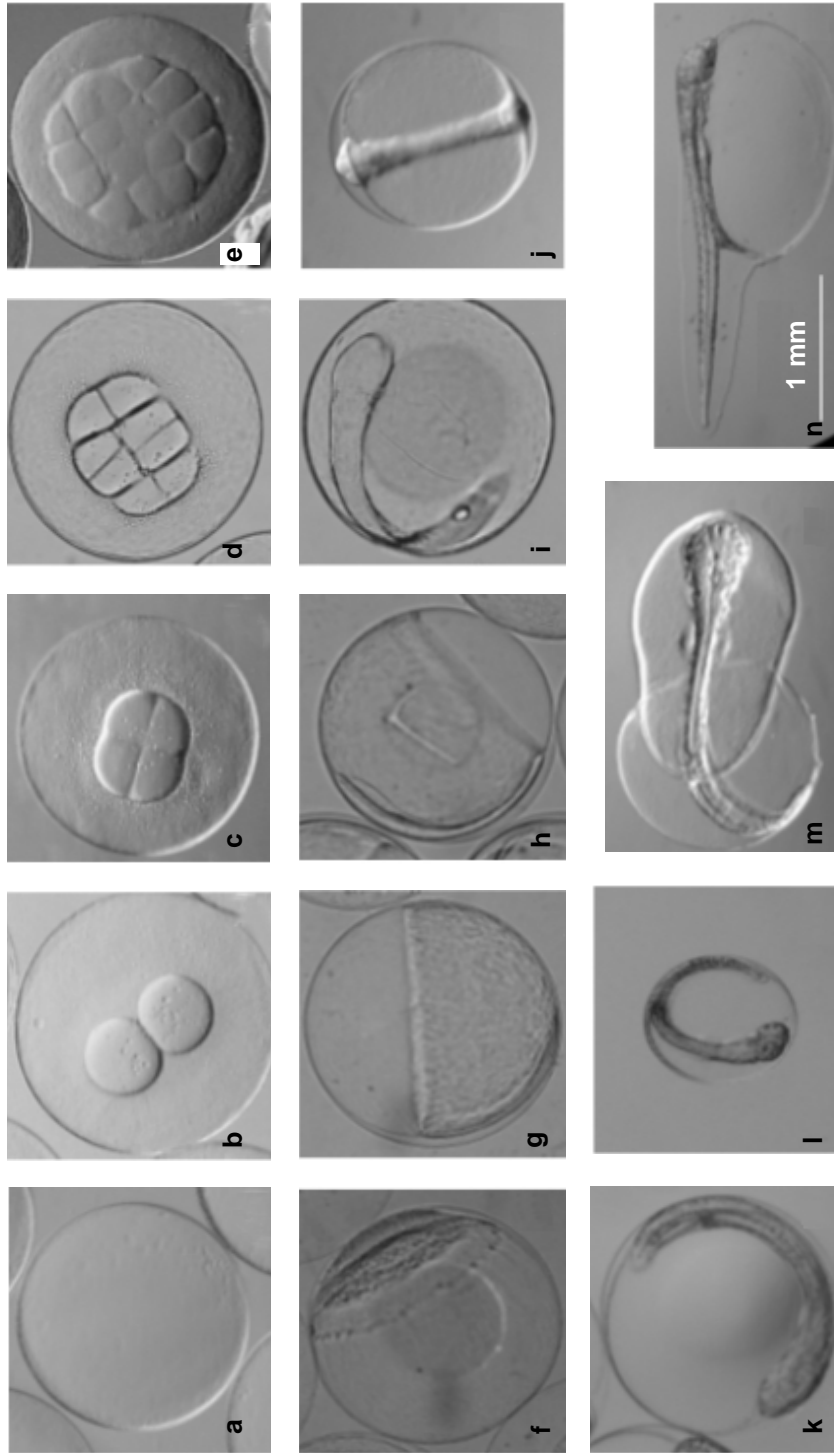


Fig. 3. Embryonic development of *Pleuronectes flesus luscus* at 10.1°C. a = fertilized egg; b = 2-cell stage, 2 h after insemination; c = 4-cell stage, 3 h 50 min; d = 8-cell stage, 4 h 40 min; e = 16-cell stage, 5 h; f = blastula, 22 h 30 min; g = gastrula, 23 h; h = formation of embryo, 31 h; i = appearance of Kupffer's vesicle, 46 h 30 min; j-k = formation of otocyte, melanophores appeared on the embryo, 70 h 30 min; l = embryo begins to move, 76 h, disappearance of Kupffer's vesicle, 92 h 30 min; m = beginning of hatching, embryo fully formed emerging from eggshell, 112 h; n = newly hatched larvae ranging 2.56-3.20 mm total length (mean 2.77±0.104 mm), with unpigmented eyes, unformed mouth, closed anus; melanophores distributed on central notochord, 117 h.

present study, increasing the incubation temperature from 10.1°C to 13.5°C reduced the incubation time as per the formula of Hamel et al. (1997). The model provided a simple and reliable tool for the aging of flounder eggs within the viable temperature range (Bermudes and Ritar, 1999).

Eggs of *P. flesus luscus* are similar to those of most marine fishes. They are pelagic and float individually near the surface, are spherical with a diameter of approximately 1 mm, and hatch into undeveloped yolk-sac larvae (Cerqueira, 2005). The average egg diameter is slightly larger than in *P. orbignyanus* (0.818 mm; Bambill et al., 2006) and *R. plebeia* (0.62 mm; Colman, 1973), but within the ranges reported by Zaharia et al. (2000) and Sahin (2000) for *P. flesus luscus* (1.04-1.30 and 1.15 mm, respectively).

Egg development in *P. flesus luscus* was very similar to egg development in *P. maxima* as described by Ciftci et al. (2002; egg diameter 1.08-1.21 mm). First segmentation occurred in almost the same time intervals after fertilization, and hatching time was about 110 h at 14-15°C. Zaharia et al. (2000) reported that the embryonic development of *P. flesus luscus* lasted 5-6.5 days at 4-14°C. Hatching of *P. orbignyanus* eggs occurred after an incubation period of 40-50 h at temperatures ranging 18-20°C (Cerqueira, 2005). The time to hatching in our study concur with those of Ciftci et al. (2002) and Zaharia et al. (2000).

In conclusion, the present study demonstrated that broodstock management and artificial spawning in captivity can be achieved in adult flounder, *P. flesus luscus*, obtained from the wild. Egg production on a commercial scale seems possible, but larvae production and on-growing need further study.

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